Virology 289, 63–73 (2001)
doi:10.1006/viro.2001.1088, available online at http://www.idealibrary.com on IDEAL®

Rubella Virus DI RNAs and Replicons: Requirement for Nonstructural Proteins Acting *in Cis* for Amplification by Helper Virus

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Received May 25, 2001; returned to author for revision June 13, 2001; accepted July 16, 2001

A rubella virus (RUB) replicon was constructed by replacing the 3′ proximal structural protein ORF (SP-ORF) in Robo402, a RUB infectious cDNA clone, with a reporter gene, green fluorescent protein (GFP). This replicon, RUBrep/GFP, mimics naturally occurring RUB defective-interfering (DI) RNAs generated during serial undiluted passage that maintain the 5′ proximal nonstructural protein ORF (NS-ORF) but contain deletions in the SP-ORF. Following transfection of Vero cells with *in vitro* RNA transcripts from RUBrep/GFP, replicon replication occurred and the replicon was amplified and spread to other cells in the presence of standard helper virus. GFP expression was a much more sensitive indicator of replicon replication than was Northern analysis to detect replicon-specific RNAs. Most of a series of RUBrep/GFP constructs with deletions in the NS-ORF not only were incapable of self-replication, but were not amplified by standard helper virus. The only exception was a construct with an in-frame deletion between two *Not*I sites that removed nucleotides 1685–2192 of the genome; this construct did not express GFP by itself, but did express GFP in the presence of standard helper RUB and was spread to other cells. Thus, with the exception of this region, the NS-ORF is required *in cis* for amplification of RUB replicons by standard helper virus, explaining the selection of DI RNAs that maintain the NS-ORF. Surprisingly, when the *Not*I deletion was introduced into Robo402, a viable virus resulted that replicated only threefold less efficiently than did Robo402 virus. Thus, the *Not*I region of the NS-ORF is not necessary for virus replication. This deletion covers a region of the NS-ORF without predicted function, which therefore may function as a spacer or hinge between functional domains. Nevertheless, it was an unexpected finding that a small virus such as RUB could dispense with ~10% of its genome.

INTRODUCTION

Rubella virus (RUB) is the etiologic agent of a disease of humans known as rubella or German measles. RUB is the sole member of the *Rubivirus* genus in the *Togaviridae* family of animal viruses (for a review, see Frey, 1994). The genome of RUB consists of a single-stranded, plus-polarity RNA of 9762 nucleotides (nts) in length and contains two long, nonoverlapping open reading frames (ORFs). The genomic RNA, which is infectious, is capped at the 5′ terminus and polyadenylated at the 3′ terminus. In infected cells, the genomic RNA functions as an mRNA to produce a polyprotein precursor encoded by an ORF at the 5′ end of the genome (the nonstructural or NS-ORF) that is processed by posttranslational cleavage into the two viral nonstructural proteins, P150 and P90. These NS proteins function in virus RNA replication; P90 contains the RNA-dependent RNA polymerase (RDRP). The genomic RNA then serves as a template for synthesis by the viral RDRP of a full-length minus strand complementary to the genomic RNA. This minus-strand RNA is then used as a template for synthesis of more positive strands. In addition to the genomic RNA, transcription initiating at an internal site on the minus-strand template leads to production of a 3327-nt subgenomic (SG) mRNA that is colinear with the 3′-terminal one-third of the genomic RNA. The initiation site for SG RNA synthesis is between the ORFs and is termed the SG promoter. Translation of the SG mRNA yields the three viral structural proteins, the capsid protein (C) and the two envelope glycoproteins (E1 and E2), which are encoded by a 3′ proximal ORF (the structural or SP-ORF). Processing of the structural proteins from the polyprotein precursor is mediated by the cellular enzyme, signal endopeptidase.

In addition to protein coding regions, viral genomes contain nucleotide sequences required for recognition by the proteins involved in genome replication and packaging. In some cases, these *cis*-acting, regulatory sequences overlap with protein coding regions, making them difficult to identify and study using genomic infectious cDNA vectors. A frequently used alternate approach has been to utilize defective interfering (DI) RNAs (Levis *et al.,* 1986, 1990; White *et al.,* 1998). DI RNAs are produced by nearly all viruses and contain partially deleted genomes that are spontaneously generated during

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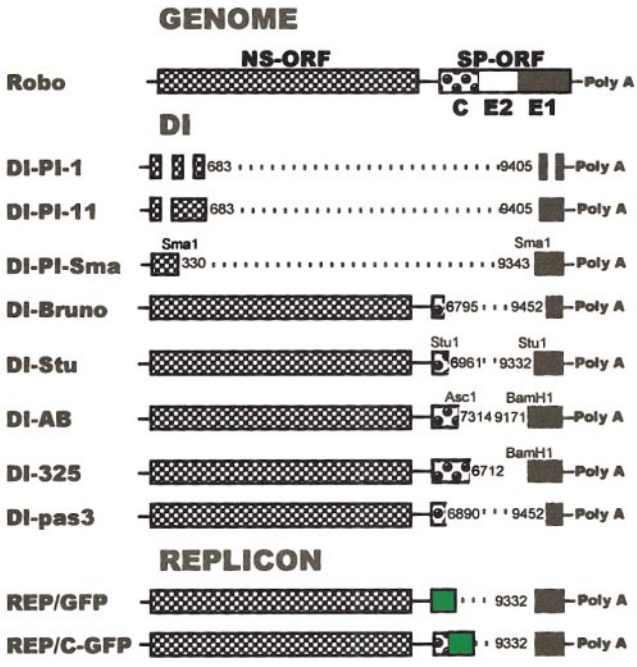

FIG. 1. Genomic, DI, and replicon infectious cDNA constructs. The RUB genomic map, as found in the Robo302/402 genomic infectious cDNA clones, is shown at the top of the diagram. ORFs are shown as boxes (NS, nonstructural; SP, structural protein containing the virion proteins C, E2, and E1) and UTRs as lines. Below the genomic infectious clone are eight DI infectious cDNA constructs. Deletions in these constructs are denoted by dashed lines; the numbers on either side of the dashed lines are the nucleotide positions of the genome at which the deletions begin and end. Of the DI constructs, the first three (DI-PI) represent the sequence pattern of DI RNAs characterized from persistent infection: DI-PI-1 and -11 were RT-PCR amplified from persistently infected cells while DI-PI-Sma was created using convenient restriction sites. The two small deletions within the 5′ genomic sequence in DI-PI-1 are between nts 86 and 267 and between nts 411 and 537 while the deletion at the 3′ end is between nts 9450 and 9624. The 5′ deletion in DI-PI-11 is between nts 9450 and 9624. The 5′ deletion in DI-PI-11 is between nts 86 and 267. The bottom five DI constructs represent the sequence pattern of DI RNAs generated during serial undiluted passage. Of these, DI-Bruno and DI-P3 were RT-PCR amplified from serially passaged virus stocks that contained DI RNAs, DI-Stu and DI-AB were created using convenient restriction sites, and DI-325 was engineered to contain the entire C gene. Of the two replicon constructs, in RUBrep/GFP the GFP gene follows the 5′ UTR of the SG RNA and a short multicloning site sequence (Pugachev *et al.,* 2000) while in RUBrep/C-GFP the GFP gene was fused in-frame with the C gene at the *Stu*I site at nt 6963.

viral genome replication. Due to the deletions, most DI RNAs fail to replicate autonomously and require coinfection with standard helper virus to provide *trans*-acting proteins. However, DI RNAs must retain the *cis*-acting elements. Therefore, DI cDNA constructs are commonly used in place of genomic infectious cDNA vectors since they are smaller and mutations can be generated without regard for coding sequences. Recently, DI RNA constructs that express reporter proteins have been developed that simplify assay procedures since reporter gene expression correlates with DI RNA replication (Barclay *et*

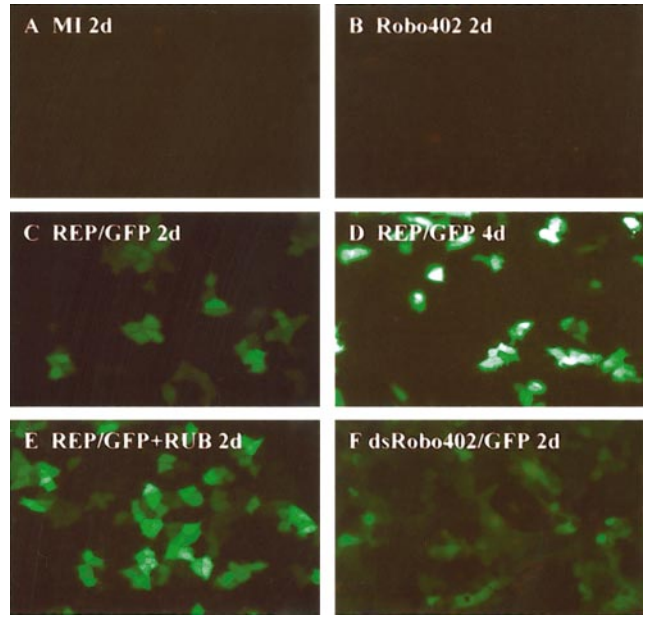

FIG. 2. GFP expression in RUBrep/GFP-transfected cells. Vero cells were mock-transfected (A) or transfected with *in vitro* transcripts from Robo402 (B), RUBrep/GFP (C, D, and E), or dsRobo402/GFP (F). The cells in E were infected with RUB (m.o.i. ~ 1 PFU/cell) 24 h prior to transfection. At 2 days (A, B, C, E, and F) or 4 days (D) posttransfection, cells were inspected for GFP expression and micrographs were taken using a Nikon E800 microscope with a Zeiss Axiocam.

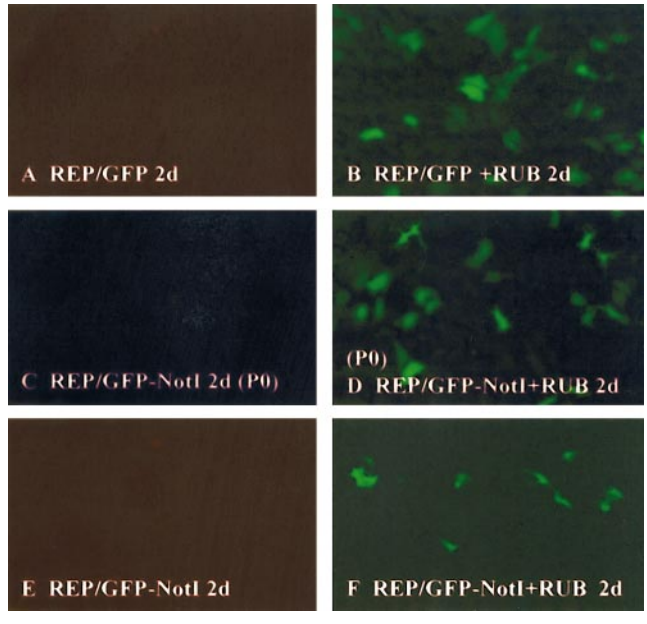

FIG. 3. GFP expression following passage of replicons. Vero cells mock-infected or infected with RUB (m.o.i. ~ 1 PFU/cell) were transfected 24 h later with *in vitro* transcripts from RUBrep/GFP or RUBrep/GFP-*Not*I. Two days posttransfection, half of the culture fluid was harvested and used to infect fresh Vero cells. Cells were inspected for GFP expression 2 days posttransfection (P0) or passage (P1). (A) P1 from mock-infected, RUBrep/GFP-transfected cells; (B) P1 from RUB-infected, RUBrep/GFP-transfected cells; (C) mock-infected, RUBrep/GFP-*Not*I-transfected cells (P0); (D) RUB-infected, RUberp/GFP-*Not*I-transfected cells (P0); (E) P1 from mock-infected, RUBrep/GFP-*Not*I-transfected cells; (F) P1 from RUB-infected, RUBrep/GFP-*Not*I-transfected cells.

al., 1998; Frolov *et al.,* 1999; Izeta *et al.,* 1999; Khromykh and Westaway, 1997; Levis *et al.,* 1987; Liao and Lai, 1994; Molenkamp *et al.,* 2000; Percy *et al.,* 1992).

DI RNAs generated during serial undiluted passage of RUB uniformly maintain the 5′ 75% of the genome, extending from the 5′ end through the NS-ORF, subgenomic promoter, and 5′ end of the SP-ORF (Derdeyn and Frey, 1995). Internal deletions of the SP-ORF are present, but the 3′ 300 nts of the genome are retained. These DI RNAs thus synthesize a truncated SG RNA. Interestingly, the genomic structure of these DI RNAs resembles "replicon" constructs that have been developed for alphaviruses, the other Togavirus genus (reviewed in Schesinger and Dubensky, 1999). In an alphavirus replicon, the SP-ORF is replaced with a foreign gene. Upon introduction into a cell, replication of the replicon genome occurs along with foreign gene expression; however, packaging and cell-to-cell spread do not occur unless a source of virion structural proteins is present. In culture cells persistently infected with RUB, DI RNAs of 1000–3000 nts accumulate (Frey and Hemphill, 1988; Abernathy *et al.,* 1990). The generation and replication of these DI RNAs have not been studied in any detail.

The goal of this study was to develop DI RNA-based vectors for use in studying the *cis*-acting elements in the RUB genome. We have previously used a RUB genomic infectious cDNA vector to study the *cis*-acting elements at both the 5′ and 3′ ends of the genome (Pugachev and Frey, 1998; Chen and Frey, 1999). However, in both cases, these elements overlap the ORFs. Considering the genomic similarity between the RUB DI RNAs generated during serial undiluted passage and alphavirus replicons, we constructed a RUB replicon based on the alphavirus model, which proved much more amenable to analysis due to its expression of a reporter gene. In a subsequent study, we found that the NS-ORF is required in *cis* amplification in the presence of standard helper virus as well as replicon replication. An exception was a 507-nt region of the NS-ORF that was not needed for amplification and was also dispensable for standard virus replication. These results were unexpected since (i) the NS-ORF is not required *in cis* for amplification of alphavirus DI RNAs in the presence of standard helper virus (Monroe and Schlesinger, 1984) and (ii) ~10% of the NS-ORF is not necessary for virus replication.

RESULTS

RUB DI and replicon constructs

The cDNA constructs based on RUB DI RNAs are shown in Fig. 1. In a previous study in our laboratory, preliminary characterization was done on a RUB persistently infected Vero cell line that had been maintained for over 3 years (Derdeyn, 1994). The predominant virus-specific intracellular RNAs in these cells were DI RNAs that migrated as a diffuse band of between 2000 and 3000 nts. Since this band hybridized to oligonucleotide probes from both the 5′ and 3′ ends of the RUB genome, RT-PCR was performed using template intracellular RNA from this cell line and primers from the 5′ and 3′ ends of the genome. The RT-PCR products were between 800 and 2000 nts in length and contained large internal deletions of the genome. Constructs based on two of these amplification products (DI-PI-1 and DI-PI-11) plus a third that fused the 5′ and 3′ ends of the genome using *Sma*I restriction sites (DI-PI-Sma) were generated. However, when transcripts from these constructs were used to transfect Vero cells in the presence of standard helper virus, negative-strand RNA corresponding to any of the three constructs could not be convincingly detected by either Northern hybridization or RT-PCR (data not shown). Interestingly, in cells transfected with transcripts from either of the constructs from naturally occurring DI RNAs (DI-PI-1 and -11), genomic and subgenomic RNA synthesis by the standard helper virus was markedly inhibited.

We then made a series of constructs based on deletions in the SP-ORF either detected in naturally occurring DI RNAs generated during serial undiluted passage (DI-Bruno; Derdeyn and Frey, 1995) or generated using convenient restriction sites within the SP-ORF (DI-Stu, DI-AB, and DI-325). However, when transcripts from these constructs were used to transfect Vero cells that had been coinfected with standard helper virus and passaged up to four times, construct-specific RNAs were not detectable (data not shown). In some of these experiments, a DI RNA of ~9000 nts was detected by passage 2 in cells infected with standard helper virus alone as well as cells coinfected with standard helper virus and *in vitro* transcripts. Since previously DI RNA generation had not been detectable prior to passage 4, this species appeared to be particularly efficient in replication and therefore the SP-ORF of this species was characterized by RT-PCR amplification. The RT-PCR amplification product contained a deletion extending between nts 6890 and 9452; this deletion was introduced into Robo302 to create a construct termed DI-P3. To test its replication efficiency, Vero cells were electroporated with Robo302 transcripts (to suppress *de novo* DI RNA generation) alone or in combination with DI-325 or DI-P3 transcripts. By Northern hybridization, the DI genomic and subgenomic RNA species were detected in cells cotransfected with Robo302 and DI-P3 transcripts (data not shown), indicating that this construct was functional. However, DI-specific RNAs were not detectable in cells electroporated with Robo302 transcripts alone or Robo302 plus DI-325 transcripts, confirming that the DI-325 construct was nonviable. DI-specific RNA species were also not detected in cells transfected with DI-325 or DI-P3 transcripts alone, indicating that standard helper genomes were necessary to amplify DI RNA synthesis to detectable levels.

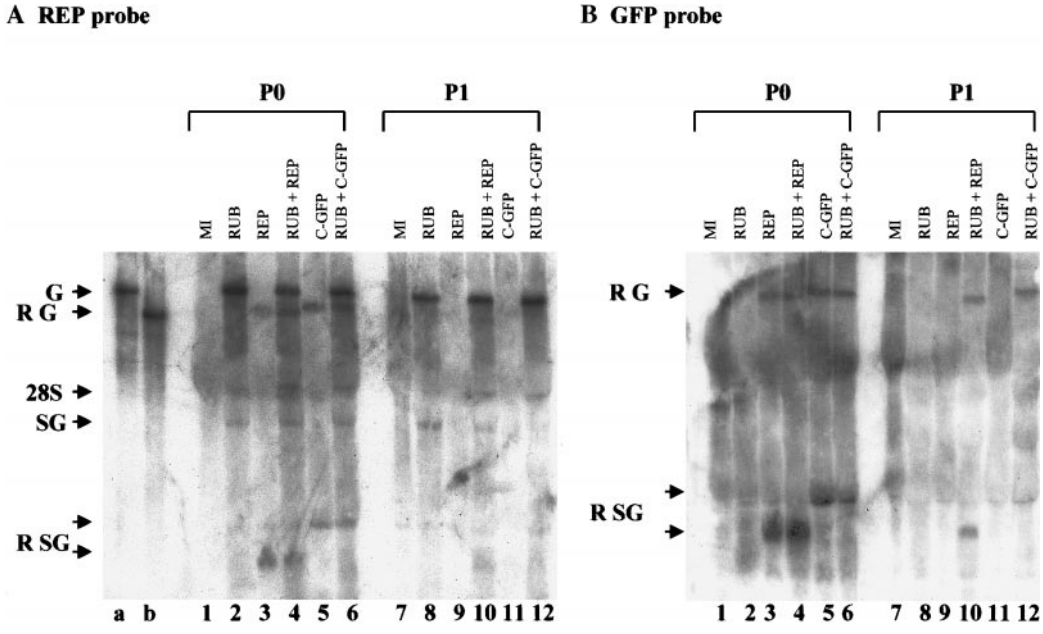

FIG. 4. Detection of replicon-specific intracellular RNA species. Mock-infected (lanes 1, 3, and 5) or RUB-infected (m.o.i. ~ 1 PFU/cell, infection 24 h prior to transfection; lanes 2, 4, and 6) Vero cells were mock-transfected (lanes 1 and 2) or transfected with transcripts from RUBrep/GFP (lanes 3 and 4) or RUBrep/C-GFP (lanes 5 and 6). The culture medium was harvested and total intracellular RNA was extracted 2 days posttransfection/infection for infected cells and 4 days posttransfection for mock-infected cells. Half of the culture medium was used to infect fresh cells; cells infected with culture medium from infected cells were preinfected with RUB (m.o.i. ~ 1 PFU/cell) 24 h previously. Total intracellular RNA was extracted from all these cells 2 days postpassage. Passage: Lane 7, mock-infected; Lane 8, RUB-infected; Lane 9, RUBrep/GFP-transfected; Lane 10, RUBrep/GFP-transfected, RUB-infected; Lane 11, RUBrep/C-GFP-transfected; Lane 12, RUBrep/C-GFP-transfected, RUB-infected. Duplicate Northern blots of these intracellular RNAs were prepared and one was probed with ^{32}P-nick-translated RUBrep/GFP (A) while the other was probed with ^{32}P-nick-translated pGEM/GFP. Lanes a and b in A contain transcripts from Robo402 and RUBrep/GFP, respectively. The positions of migration of the genomic (G), subgenomic (SG), replicon (R), and replicon subgenomic (R-SG) RNAs are indicated. It is to be noted that RUBrep/C-GFP contains C gene sequences and thus both its genomic and SG RNAs are larger than those of RUBrep/GFP. The position of migration of cell 28S RNA, which causes a blob in the Northern blots, is also indicated.

As a more rapid assay of DI RNA replication, a replicon was generated in which the reporter GFP gene replaced the majority of the SP-ORF (Fig. 1). As shown in Figs. 2C and 2D, in Vero cells transfected with transcripts from this replicon construct (RUBrep/GFP) GFP expression was readily detectable. The intensity of GFP expression was comparable to or greater than expression by the double-subgenomic infectious cDNA construct, dsRobo402/GFP (Fig. 2F), depending on the time post-transfection that GFP fluorescence was assayed. Because of the limited titers of RUB infecting stocks (~10^7 PFU/ml) and the nonsynchronous infection initiated even at high m.o.i.'s, amplification of the replicon by standard helper RUB was studied by infecting Vero cells with RUB (m.o.i. ~1 PFU/cell) 24 h prior to transfection with RUBrep/GFP transcripts. As shown in Fig. 2E, GFP fluorescence was more widespread in RUBrep/GFP-transfected, RUB-infected cells than in cells transfected with RUBrep/GFP alone. When culture fluid was harvested and passaged to fresh Vero cells, no GFP expression was detected in cells infected with culture fluid from RUBrep/GFP-transfected cells (Fig. 3A), but GFP expression was detected in cells infected with culture fluid from RUBrep/GFP-transfected, RUB-infected cells (Fig. 3B). In total, these results indicate that the replicon construct is capable of self-replication, including expression of the GFP gene, and could be amplified, packaged, and spread in the presence of helper virus. To determine the effect of the 5′ end of the C gene retained in the naturally occurring DI RNAs, a replicon construct, RUBrep/C-GFP, was generated in which the C gene through nt 6963 (a *Stu*I site; 152 aa of the C protein) was fused in-frame with GFP (Fig. 1). Results of GFP expression by this replicon in the absence and in the presence of standard helper RUB were similar to results obtained with the RUBrep/GFP replicon without the additional C gene sequences (data not shown).

Northern analysis was used to study virus-specific RNA synthesis in replicon-transfected cells. As shown in Fig. 4A, in cells transfected with either RUBrep/GFP or RUBrep/C-GFP, replicon-specific genomic and SG RNAs were detected both in the absence and in the presence of standard helper virus (Fig. 4A, lanes 3 and 5 vs lanes 4 and 6). The amount of replicon RNA produced by the two constructs was similar (Fig. 4A, lanes 3 and 4 vs lanes 5 and 6) and there was no evidence of interference

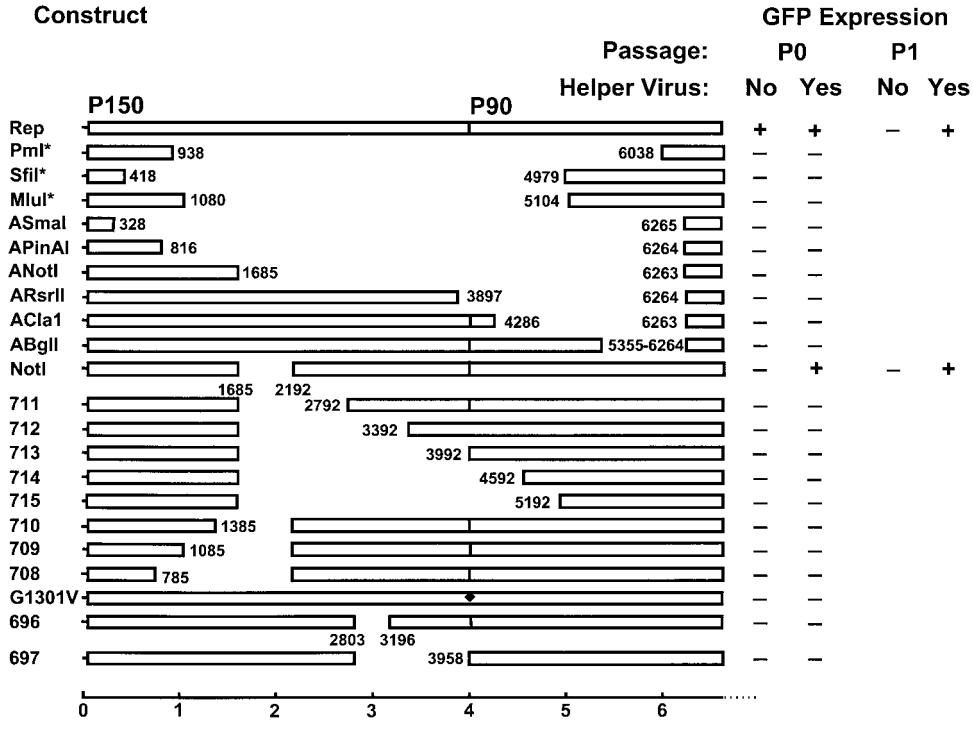

FIG. 5. NS-ORF deletions in RUBrep/GFP. Shown are a series of mutations, all but one deletions, made in the NS-ORF of RUBrep/GFP. The scale at the bottom is in kilobases from the 5′ end of the RUB genome and the 5′ and 3′ breakpoints of each deletion are given (where no number is given, it is the same as the construct immediately above). All of these deletions were in-frame with the exception of the three marked with an asterisk. In the G1301V mutation, the G at the P1 position of the NS protease cleavage site was mutated to V. Transcripts from these constructs were used to transfect uninfected Vero cells or Vero cells that had been infected with helper RUB (m.o.i. ~ 1 PFU/cell, infection 24 h before transfection). Cultures were inspected for GFP expression 2 days after transfection. From cultures that exhibited GFP expression, half of the culture medium was passaged to fresh Vero cells and cultures were inspected for GFP expression 2 days after passage.

with the synthesis of the standard helper virus RNA species (Fig. 4A, lane 2 vs lanes 4 and 6). Although the amount of replicon-specific RNA was comparable in the absence or in the presence of standard helper virus, a direct comparison cannot be made because RNA was extracted at 2 days posttransfection from cells infected with standard helper virus and at 4 days posttransfection from cells that did not receive standard helper virus; however, replicon spread was much greater in cells infected with standard helper virus as shown in Fig. 2. Following passage of the transfected culture medium to fresh Vero cells, replicon-specific RNAs were not detected, even though they were expected to be present in cultures infected with standard helper virus since GFP expression was detected following passage (Fig. 2). It was reasoned that this was likely due to the low titers of both replicons and standard helper virus produced. Therefore, to increase the amount of standard helper virus present, the Vero cells to be infected with culture medium from cells transfected with replicons in the presence of standard helper virus were preinfected with standard helper virus at an m.o.i. ~1 PFU/cell 24 h prior to passage. Even under these conditions, replicon-specific RNAs were not detectable using a RUBrep/GFP probe. However, when a GFP-specific probe was employed (Fig. 4B), the replicon genomic and subgenomic RNAs were observed. The amounts of replicon-specific RNAs produced by the two replicons following passage were similar.

Deletion studies

With RUB replicons in hand, it was next of interest to determine the minimal genomic sequences necessary for amplification by standard helper virus. To this end, a series of deletions in the NS-ORF of RUBrep/GFP was constructed using convenient restriction sites (Fig. 5). In aggregate, these deletions covered most of the NS-ORF; some of these deletions maintained the ORF while some did not. None of these deletion constructs expressed GFP in the absence of standard helper virus, and only one, a construct with an in-frame *Not*I–*Not*I deletion between nt 1685 and 2192 in the middle of the P150 gene, expressed GFP in the presence of standard helper virus (Fig. 3D). When culture fluid from RUBrep/GFP-*Not*I transfected, RUB-infected cells was passaged to fresh Vero cells, GFP expression was observed (Fig. 3F). A series of in-frame deletions was made that expanded the extent of the *Not*I–*Not*I deletion in either the 5′ or the 3′ direction (Fig. 5); however, none of these mutants ex-

pressed GFP in the absence or in the presence of standard helper virus. Additionally, GFP expression in the absence or in the presence of helper virus was not observed with a mutation in the NS protease cleavage site as well as constructs with mutations of the NS protease domain or both the protease domain and the cleavage site.

These results indicated that the NS-ORF was necessary *in cis* for self-replication of the replicon (as expected). Unexpectedly, the NS-ORF was also necessary *in cis* for amplification of the replicon by standard helper virus, with the exception of the *Not*I–*Not*I region. This indicates that the *Not*I–*Not*I region can be complemented *in trans* by standard helper virus and to test this hypothesis, the *Not*I–*Not*I deletion was then introduced into Robo402, generating Robo402/Δ*Not*I. Surprisingly, transcripts from this construct induced CPE within 2–3 days posttransfection and thus, the *Not*I–*Not*I region of the NS-ORF is not essential for virus replication. In comparison with Robo402 virus, Robo402/Δ*Not*I virus replicated to two- to threefold lower titers and produced smaller plaques.

DISCUSSION

Extensive characterization has shown that DI RNAs generated by serial undiluted passage of RUB contain deletions in the SP-ORF and this report contains the first construction of biologically active vectors based on this class of DI RNA. It is not clear why several of the DI cDNA constructs were not biologically active, although deleterious mutations in the Robo102 infectious clone (Pugachev *et al.,* 1997) or unrecognized deleterious mutations in the DI cDNA used in construction of the vectors could have been at fault. Additionally, the deletions amplified by RT-PCR from DI RNA populations may have been from inert rather than active DI RNA molecules. In this regard, the deletion in the biologically active construct DI-P3 interrupted the SP-ORF translation frame and thus maintenance of this ORF is not necessary for amplification of RUB DI RNAs. The RUBrep/GFP replicon resembled the structure of the DI RNAs generated during serial undiluted passage. With the replicon, it was shown that RUB genomic derivatives that maintain the NS-ORF, but not the SP-ORF, are capable of self-replication, as expected. To more closely conform to the DI RNA structure, a replicon that maintained a portion of the C gene fused in-frame to the GFP gene, RUBrep/C-GFP, was constructed. However, in the experiments done in this study, no difference in replication could be detected between the replicons with and without the portion of the C gene. Neither replicon interfered with standard virus replication, as do DI RNAs generated during serial undiluted passage (Frey and Hemphill, 1988).

Replicon mutants with deletions in the NS-ORF were unable to replicate, as expected. Unexpectedly, however,

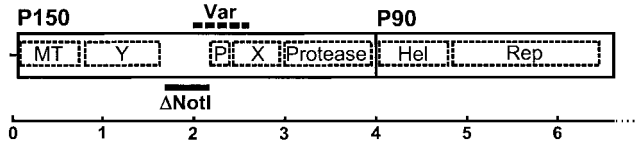

FIG. 6. The *Not*I region in relation to motifs within the RUB NS-ORF. Shown in the diagram of the NS-ORF is the *Not*I deletion, the variable region (Pugachev *et al.,* 1997), and the motifs predicted by computer-assisted homology comparison with the replicase proteins of other plus-polarity RNA viruses (Koonin *et al.,* 1992) (MT, methyl transferase; Y, Y domain; P, proline hinge; X, X domain; protease, NS protease; Hel, helicase; Rep, replicase). The genomic scale at the bottom of the diagram is in kilobases.

these mutants were also not amplified by standard helper virus, with the exception of the *Not*I–*Not*I deletion between nts 1685 and 2192 in the P150 gene. Thus, the NS-ORF is required *in cis* for both replication and amplification of the replicon. This explains the retention of the NS-ORF in DI RNAs generated during serial undiluted passage of RUB. Recently, Liang and Gillam (2001) demonstrated that negative-polarity RNA synthesis in cells transfected with transcripts from genetically tagged RUB genomic constructs was *cis* preferential. Such a *cis* requirement for replicase proteins has been documented for DIs/replicons of other plus-strand RNA virus families, namely, the picornaviruses (Hagino-Yamagishi and Nomoto, 1989). Flavivirus replicons also require several, but not all, of the nonstructural proteins *in cis* (Khromykh *et al.,* 2000). However, alphavirus DI RNAs that are efficiently amplified by helper virus contain deletions of most of both of the genomic ORFs (Levis *et al.,* 1986). The most likely explanation for requirement of the NS-ORF *in cis* is that the replicase proteins interact directly with the replicon RNA from which they are translated to initiate RNA replication.

Investigation of the NS-ORF deletions in the replicon led to identification of the *Not*I–*Not*I region that was found to be dispensable for virus replication. As shown in Fig. 6, this region encodes a hypervariable region in P150 with no function assigned by computer-assisted homology searching. Possibly, this region serves as a linker or spacer between the other functional domains of P150. Interestingly, while this region was dispensable for standard virus replication as measured by PFU production, it appeared to be necessary for replicon replication as measured by GFP expression and could be complemented by standard helper virus. The function of this region in replicon replication, as opposed to standard virus replication, needs further analysis using the same assay for replication.

One of the goals of this study was to generate stripped-down vectors for analysis of *cis*-acting sequences in the RUB genome. Because of the necessity of the NS-ORF *in cis* for amplification in the presence of standard helper virus, the vector generated is only ~2000 nts shorter than the infectious cDNA clone. The

overlap between the E1 coding sequences and the 3′ *cis*-acting elements has been eliminated (which impeded mutational characterization of these elements using the infectious clone; Chen and Frey, 1999), but in other regions of the genome overlapping functions remain. For example, when the putative packaging signal between nts 347 and 376 shown to bind RUB capsid protein *in vitro* (Liu *et al.,* 1996) was deleted from RUBrep/GFP, the replicon lost the ability to be amplified by standard helper virus, presumably because the region of the NS-ORF in which it occurs is required *in cis* (W.-P. Tzeng, unpublished observations). However, the construction of a RUB replicon that can be packaged *in trans* will be very useful for development of expression, vaccine, and gene therapy vectors as has been the case with alphavirus replicons (Dubensky and Schlesinger, 1999). RUBrep/GFP is not cytopathic in transfected Vero cells and cells transfected with the replicon can be passed at least four times while maintaining a constant level of GFP expression. On the other hand, a stock of RUBrep/GFP generated by transfection of RUBrep/GFP transcripts into cells coinfected with standard helper virus can be subjected to serial undiluted passage in Vero cells a maximum of four times before GFP expression is lost. Whether this is due to inefficiencies in packaging or in other aspects of amplification by standard helper virus is unknown. The former possibility could be resolved by determining the packaging efficiency of replicons in cells expressing the RUB structural proteins while insight into the latter possibility could be gained by studying DI RNAs that persist and become the dominant RNA species during serial undiluted passage to see whether specific genomic regions are required or whether adaptational mutations are acquired (Frey and Hemphill, 1988).

Finally, this is the first report of characterization of DI RNAs generated during RUB persistent infection. These DI RNAs appear to retain the 5′ and 3′ sequences but delete large internal regions of the genome, as would be predicted from their size of 1000–3000 nts. However, constructs based on cDNA clones amplified from persistently infected cells did not replicate in the presence of standard helper virus, although they appeared to interfere with standard helper virus replication. It is to be noted that the amplified cDNAs (~1000 nts in length) were in the size range reported for DI RNAs generated during persistent infection; they were shorter than the majority of DI RNA species present at the passage from which they were derived (2000–3000 nts). It will take more analysis to answer the most relevant question about such DI RNAs raised by the results of this study, namely, how they are amplified by helper virus without the presence of the NS-ORF *in cis.*

MATERIALS AND METHODS

Cells and viruses

Propagation of Vero cells and production and titration of stocks of the W-Therien and F-Therien strains of RUB as well as Robo402 virus were done as described previously (Pugachev *et al.,* 1997).

Plasmids and recombinant DNA manipulations

Recombinant DNA manipulations were essentially as described in Sambrook *et al.* (1989) with minor modifications. *Escherichia coli* MC1061, DH-5α, and JM109 were used as the bacterial hosts. Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Beverly, MA) or Roche Molecular Biochemicals (Indianapolis, IN) and used essentially as recommended by the manufacturers. Standard PCRs contained 400 ng of each oligonucleotide primer, 20 ng of linearized plasmid template, a 200 μM concentration of each dNTP, 5 units of *Ex-Taq* DNA polymerase (PanVera/TaKaRa, Madison, WI) in 1× buffer provided by the manufacturer in total volume of 50 μl. The amplification protocol was 35 cycles of 20 s at 98°C, 30 s at 50°C, and 2 min at 70°C, followed by 1 cycle of 10 min at 72°C. Following the reaction, PCR amplification products were purified using Wizard PCR Preps (Promega, Madison, WI) or QIAquick PCR Purification Kits (Qiagen, Valencia, CA) or digested with appropriate restriction enzymes followed by resolution by electrophoresis in 1% agarose gels in TAE buffer (40 mM Tris–acetate, 1 mM EDTA) in the presence of ethidium bromide, excision of the band, and purification using a GeneClean II Kit (Bio 101, Carlsbad, CA) or QIAquick Gel Extraction Kit (Qiagen). The genomic cDNA clones Robo12, Robo102, Robo302, and Robo402 and the double subgenomic vector expressing GFP, dsRobo402/GFP, were described previously (Wang *et al.,* 1994; Pugachev *et al.,* 1997, 2000).

DI RNAs in a RUB-persistently infected Vero cell line maintained for over 3 years were characterized by extraction of total intracellular RNA, RT-PCR with first-strand cDNA synthesis primed with oligo(dT), and amplification with two primers, 5′-CCGGAATTCCAATCCAATGGAAGCTATCGGACCTCGCTTAG-3′ [containing an *Eco*RI site (underlined) followed by the 5′ 32 nts of the RUB genome] and 5′-CCGGTCTAGACTATACAGCCAACAGGTGCGGGAATCT-3′ {containing an *Xba*I site (underlined) followed by the complement of the 3′ 27 nts of the RUB genome [preceding the poly(A) tract]}, ligation into *Eco*RI–*Xba*I restricted pGEM-3Z(f-), and sequencing. The inserts in two of the clones were excised by restriction with *Apa*I (sites at nts 289 and 9705 of the genome, which were retained in these clones) and introduced into *Apa*I-restricted Robo102 from which the internal cDNA fragments had been removed. The DI-PI-Sma vector was produced by digesting Robo102 with *Sma*I and religating

to produce a construct containing a cDNA insert extending from the 5′ end through nt 328 (the 5′-most *Sma*I site in the RUB genome) and then from nt 9344 (the 3′-most *Sma*I site) through the 3′ end. DI-Bruno was generated from a clone isolated from a cDNA library made from total intracellular RNA extracted from cells infected with virus stock that had been serially passaged 12 times and shown to contain DI RNAs (Derdeyn and Frey, 1995). This cDNA clone extended from nt 740 through the 3′ end of the genome, including a deletion between nts 6795 and 9452 in the SP-ORF. This clone was digested with *Sna*BI (unique site at nt 1207 of the genome) and *Eco*RI (linearization site at the 3′ end of the genome) and the *Sma*BI–*Eco*RI fragment containing the cDNA was used to replace the corresponding fragment in Robo12. DI-*Stu*I was produced by digesting Robo102 with *Stu*I (unique sites at nts 6963 and 9334, both within the SP-ORF) and religating while DI-AB was produced by digesting Robo102 with *Asc*I (unique site at nt 7318) and *Bam*HI (unique site at nt 9174), blunt-ending with Klenow, and religating. DI-325 was constructed by PCR amplifying a product from Robo302 template DNA with primers 5′-GAGATCTAGCCGCATGT-3′ [colinear with nts 5351 to 5346, including a unique *Bgl*II site (underlined)] and 5′-CGGGATCCCCGGCGCGCGCGGTG-3′ [*Bam*HI site (underlined) followed by sequences complementary to nts 7398 to 7411; designed to create a deletion between the 3′ end of the capsid gene and the unique *Bam*HI site at nt 9174], restricting the product with *Bgl*II and *Bam*HI, and using it to replace the *Bgl*II–*Bam*HI cDNA fragment in Robo102. To construct DI-P3, Vero cells were infected with virus stock serially passaged twice that had been shown to contain DI RNAs. Total intracellular RNA was extracted and used for RT-PCR with reverse transcription primed by 5′-ACGTGAATTCT_{20}-3′ [*Eco*RI site (underlined) followed by oligo(dT)] and amplification with this primer and 5′-GAGATCTAGCCGCATGT-3′ [colinear with nts 5351 to 5346, including a unique *Bgl*II site (underlined)]. The amplification product was restricted with *Bgl*II and *Eco*RI (linearization site at the 3′ end of the genome), subcloned, and then used to replace the *Bgl*II–*Eco*RI cDNA fragment of Robo302.

To construct the replicon RUBrep/GFP, a PCR fragment was amplified from *Eco*RI-linearized dsRobo402/GFP template with primer 5′-AGCTCACCGACCGCTACGC-3′ (nts 5321–5340 of the RUB genome; upstream from a unique *Bgl*II site at nt 5355) and primer 5′-ATAT**AGGCCT**ATGCATTATGCACATCAGTT-3′ [*Stu*I site (boldface) and *Nsi*I site (underlined) following by the 3′ terminal 14 nts of the GFP gene], restricted with *Bgl*II and *Stu*I, and ligated with *Bgl*II–*Stu*I restricted Robo402. By using this approach, the unique sites on either side of the GFP gene in dsRobo402/GFP (*Xba*I and *Nsi*I) were preserved. To construct RUBrep/C-GFP, in which the N-terminal 152 aa of the C protein were fused in-frame with GFP, a three-round asymmetric PCR amplification strategy (Chen and Frey, 1999) was employed. In the first round, asymmetric PCR using mutagenic primer 5′-GCACCCACCGAGTCTAGAGCCTGCGTGACC-3′ [nts 6953 to 6976 of the genome with an *Xba*I site (underlined) inserted between nts 6964 and 6965] *Pst*I-linearized Robo402 template was used to synthesize a single-stranded DNA (ssDNA). This ssDNA product was used as a template for a second round of asymmetric PCR primed with 5′-GAAGCGGATGCGCCAAGG-3′ (complementary to nts 7328 to 7345 of the genome) to asymmetrically amplify a complementary strand to the first-round PCR product. In the third round, *Pst*I-linearized Robo402 template was combined with the second-round PCR product and 5′-ACTAATGCATCGCCCCTGTACGTGGGG-3′ [*Nsi*I site (underlined) followed by nts 6392 to 6408 of the genome]. After digestion with *Nsi*I and *Xba*I, this fragment was included in a three-fragment ligation with the *Xba*I–*Eco*RI fragment of RUBrep/GFP (containing the GFP gene through the *Eco*RI linearization site) and the *Eco*RI–*Nsi*I fragment of NRobo402 (a Robo402 derivative in which an *Nsi*I site was placed immediately downstream from the NS-ORF). This latter fragment contains the pBR322 vector sequences and the 5′ end of the RUB genome through the NS-ORF.

A number of internal deletion mutations in the NS-ORF of RUBrep/GFP were created using restriction enzymes with two sites within the NS-ORF, namely, *Not*I (nts 1685–2192), *Pml*I (nts 938–6038), *Sfi*I (nts 418–4979), and *Mlu*I (nts 1080–5104). RUBrep/GFP DNA was restricted with one of these enzymes, the internal fragment was removed by agarose gel electrophoresis, and the vector DNA was recovered and self-ligated. A 3′ nested set of internal deletion mutants within the NS-ORF was created using a common anchor sequence near the 3′ end of the ORF and restriction sites across the ORF. For example, the deletion based on the unique *Pin*AI site at nt 816 of the genome (construct A*Pin*AI) was created by generating a PCR product with upstream primer 599 (Table 1) consisting of a *Pin*AI site followed by nts 6264–6278 of the genome and downstream primer 679 consisting of *Stu*I and *Nsi*I sites followed by the 3′ terminal 20 nts of the GFP gene on *Eco*RI-linearized dsRobo402/GFP template. The amplification product was restricted with *Pin*AI and *Stu*I and ligated into *Pin*AI(816)–*Stu*I(9334) restricted Robo402. The same strategy was employed to generate other internal deletion mutants based on the *Sma*I site (nt 328; primer 609), the *Not*I site (nt 1685; primer 616), the *Rsr*II site (nt 3897; primer 659), the *Cla*I site (nt 4286; primer 660), and the *Bgl*II site (nt 5355; primer 661). To make deletion mutants that extended the 3′ terminus of the *Not*I deletion in RUBrep/GFP-*Not*I (711, 712, 713, 714, and 715), mutagenic primers were designed to contain a *Not*I restriction site and 15 nts downstream from the desired deletion site (primers 711, 712, 713, 714, and 715, respectively; Table 1). For each construct, a PCR product was amplified from *Eco*RI linearized Robo402 template

TABLE 1

Primers Used in Construction of NS-ORF Deletion Mutations

Oligo sequence (restriction site)[a]	Genomic nts (polarity)[b]
Common-anchor restriction site deletions	
Upstream primers	
599 ATATACCGGTTCCTCGCTATCGTGC (*Pin*AI)	6264–6278 (+)
609 GCATCCCGGGCCTCGCTATCGTG (*Sma*I)	6265–6277 (+)
616 GCATGCGGCCGCGTCCTCGCTATCGTG (*Not*I)	6263–6277 (+)
659 ATATCGGACCGCCTCGCTATCG (*Rsr*II)	6264–6275 (+)
660 CCATCGATGTCCTCGCTATCG (*Cla*I)	6263–6275 (+)
661 GAAGATCTTCCTCGCTATCG (*Bgl*II)	6264–6275 (+)
Downstream primer (common)	
679 ATAT**AGGCCT**ATGCATTATGCACATCAGTT (*Stu*I *Nsi*I)	3′ 14 nts of GFP (−)
3′ extensions of *Not*I–*Not*I deletion	
Upstream primers	
711 GCATGCGGCCGCCGCGCTAGCCGCCGC (*Not*I)	2800–2814 (+)
712 GCATGCGGCCGCGCGGTGCACCCCCTC (*Not*I)	3400–3414 (+)
713 GCATGCGGCCGCGGTGCGCCGCCTCGG (*Not*I)	4000–4014 (+)
714 GCATGCGGCCGCCCGCCTTCACGAGGC (*Not*I)	4600–4614 (+)
715 GCATGCGGCCGCTTTTCGCCAGATCCC (*Not*I)	5200–5214 (+)
Downstream primer (common)	
643 GCGCGGATCCGGCAGTTATTGGCGTAGTGT	4387–4403 (−)
5′ extensions of *Not*I–*Not*I deletion	
Upstream primer (common)	
245 ACCGCCCTGGCCGAGAC	511–527 (+)
Downstream primers	
708 GCATGCGGCCGCGACTTGGTAAAGCG (*Not*I)	700–714 (−)
709 GCATGCGGCCGCCGCGTCGGGGCTCAG (*Not*I)	1070–1084 (−)
710 GCATGCGGCCGCGTCCTGTTCCTACTC (*Not*I)	1370–1384 (−)

[a] The sequences of oligonucleotide PCR primers used in construction of three sets of NS-ORF deletion mutations in RUBrep/GFP are given. With respect to the RUB genomic sequence, upstream primers are 5′ proximal and in the positive polarity while downstream primers are 3′ proximal and in the negative polarity.

[b] Nucleotides of the RUB genome encompassed by the oligonucleotide primer. In the case of primers with restrictions site, the RUB genomic sequences begin after the restriction site.

with one of these mutagenic primers as the upstream primer and downstream primer 643 (complementary to nts 4387–4403; downstream from the unique *Cla*I site at nt 4286). The amplification product was digested with *Not*I and *Cla*I and ligated with *Not*I–*Cla*I restricted RUBrep/GFP. To generate deletion mutants that extended the 5′ terminus of the *Not*I deletion in RUBrep/GFP-*Not*I (708, 709, and 710), mutagenic primers complementary to the RUB genome were designed to contain a *Not*I restriction site and 15 nts ending at the desired deletion site (primers 708, 709, and 710, respectively; Table 1). One of these mutagenic primers was used as the downstream primer with upstream primer 245 (nts 511 to 527 of the RUB genome, upstream from the unique *Bsu*36I site at nt 500) in a PCR with *Eco*RI-linearized Robo402 template. The PCR amplification products were digested with *Bsu*36I and *Not*I and ligated with *Bsu*36I–*Not*I restricted RUBrepGFP.

To generate a protease cleavage site mutant (G1301V) in RUBrep/GFP in which the G residue at the P1 position of the cleavage site (at amino acid 1301 of the NS-ORF) was replaced with V, a *Bsu*36I–*Bgl*II fragment was removed in pMAL-NSP* and a maltose-binding protein–NS–protease construct containing the desired mutation (G1301V) (Liu *et al.,* 2000) was used to replace the *Bsu*36I–*Bgl*II restriction fragment of RUBrep/GFP. To construct the protease domain deletion RUBrep/GFP-696 and the protease domain and cleavage site deletion mutant RUBrep/GFP-697, a fragment was amplified by PCR using primer 5′-ATATGCTAGCGCTTGCGGTCCCCCTG-3′ [*Nhe*I site (underlined) followed by nts 3916–3931 of the genome] or primer 5′-ATATGCTAGCGACCGACGAGGGGCTG-3′ [*Nhe*I site (underlined) followed by nts 3957–3973 of the genome], respectively, and primer 5′-GCGCGGATCCGGCAGTTATTGGCGTAGTGT-3′ (complementary to nts 4387 to 4403 of the RUB genome, a region downstream from the unique *Cla*I site at nt 4286) with *Eco*RI-linearized Robo402 template. The am-

plification product was restricted with *Nhe*I and *Cla*I and ligated with *Nhe*I–*Cla*I restricted RUBrep/GFP produced by partial digestion with *Nhe*I and *Cla*I and gel isolation of the 10.6-kb *Nhe*I(nt 2803)–*Cla*I fragment.

To produce Robo402Δ*Not*I, the *Bsu*36I–*Bgl*II fragment in RUBrep/GFP-*Not*I spanning the *Not*I deletion was used to replace the corresponding fragment in Robo402.

All mutations were confirmed by sequencing using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) and appropriate primers. Sequencing reaction products were purified on Centrisep columns (Princeton Separations, Adelphia, NJ) and cycle sequencing was performed using an ABI 373 sequencer (Perkin–Elmer Corp., Foster City, CA).

In vitro transcription and transfection

For each construct, two independent clones of each construct in which the presence of the desired mutation was verified by sequencing were used for *in vitro* transcription and transfection to confirm the phenotypes. Plasmids were purified by isopycnic centrifugation in CsCl gradients prior to *in vitro* transcription and transfection. *In vitro* transcriptions with SP6 RNA polymerase in the presence of cap analog were done as previously described (Pugachev *et al.,* 2000); with all of the constructs, linearization prior to transcription was with *Eco*RI. Vero cells in 60-mm^2 dishes were mock-infected or infected with RUB (m.o.i. ~1 PFU/cell) 24 h prior to transfection with 3–4 μg of transcripts using Lipofectamine or Lipofectamine 2000 reagent (Gibco BRL, Rockville, MD). Following transfection, the transfection solution was removed and replaced with maintenance medium. GFP expression was observed by removing the medium, adding a minimal amount of PBS to keep the cells hydrated, covering the cells in the center of the dish with a coverslip, and examining the cells with a Zeiss Axioplan microscope with epifluorescence capability. Using RUBrep/GFP transcripts to transfect mock-infected Vero cells, the efficiency of transfection was found to be roughly 6% with Lipofectamine and 34% with Lipofectamine 2000. Micrographs were taken using a Nikon E800 microscope with a Zeiss Axiocam. For passage, the medium was harvested and one-half was used to infect fresh Vero cells. Occasionally, cells were preinfected with RUB (m.o.i. ~ 1 PFU/cell) 24 h prior to passage. Where indicated, transfection was done by electroporation: ~5 × 10^6 cells in 0.4 ml of PBS were mixed with transcripts in a Gene Pulser cuvette and pulsed twice with a Bio-Rad Gene Pulser apparatus (resistance = infinity; CAP = 25 μFD; voltage = 1.5 kV with a time constant of 0.7 to 0.8 s) followed by plating in a 60-mm^2 dish.

RNA analysis

Total intracellular RNA was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH) in accordance with the manufacturer's protocol. RNA from a 35-cm^2 plate was resuspended in 40 μl of diethylpyrocarbonate-treated water and concentrations were determined spectrophotometrically using an extinction coefficient at 260 nm of 0.022 for 1 μg/ml. RT-PCR and sequencing of the PCR product were done to confirm maintenance of the *Not*I deletion in both RUBrep/GFP-*Not*I and Robo402Δ*Not*I. Fifty nanograms of primer 137 (5′-TTGACCACGACCTTGCA-3′; complementary to nts 2535–2550 of the RUB genome) was annealed with one-fifth of the extracted RNA in Superscript reverse transcription buffer (75 mM KCl, 3 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.3) followed by addition of DTT to 10 mM, each dNTP to 1 mM, 10 units/μl of Superscript reverse transcriptase, and 1 unit/μl Rnasin (Roche Molecular Biochemicals) in a final volume of 20 μl. After incubation at 45°C for 90 min and boiling for 5 min, the cDNA was used as template for PCR amplification using primers 137 and 33 (5′-CCATTTGGGACGCCATT-3′; colinear to nts 1308 to 1324 of the RUB genome). The PCR amplification product was subsequently sequenced using primer 33.

For Northern gel analysis, equivalent amounts of RNA were denatured with Glyoxal Sample Loading Dye (Ambion, Austin, TX) for 30 min at 55°C and placed on ice immediately. The denatured RNA was electrophoresed in a 1% agarose gel made in 1× NorthernMax-Gly Gel Preparation/Gel Running Buffer (Ambion). After electrophoresis, the RNAs were transferred to a nylon membrane (MSI, Magnagraph, 0.45-mm pore diameter; Westboro, MA) by capillary action for 2 h using NorthernMax Transfer Buffer (Ambion) as the transfer medium. After transfer, the nylon membrane was either baked at 80°C for 2 h or irradiated with a 254-nm UV Crosslinker Lamp (Fisher Scientific, Pittsburgh, PA). The membrane was placed in a heat-sealable plastic bag containing NorthernMax Prehyb/Hyb Buffer (Ambion) and prehybridized for 2 h at 65°C. Nick-translated RUBrep/GFP probe or pGEM-GFP probe (0.5 to 1 μg) was radiolabeled by nick translation using a Nick Translation System Kit (Promega, Madison, WI) and 50 μCi of [α-^{32}P]dCTP (3000 mCi/mmol; New England Nuclear, Boston, MA). Half of the nick-translated DNA probe was denatured in 50% formamide at 100°C for 5 min and added to the blot in Prehyb/Hyb Buffer and hybridization was allowed to occur at 65°C overnight. After hybridization, the membrane was washed at room temperature once in Low-Stringency Wash Solution 1 (Ambion) for 10 min and twice in High-Stringency Wash Solution 2 (Ambion) for 15 min at 42°C. Washed membranes were wrapped in plastic wrap and exposed to Kodak X-ray film at −70°C between two Cronex Lightning Plus intensifying screens.

ACKNOWLEDGMENTS

The authors thank Dr. Robert Simmons for confocal imaging. This research was supported by a grant from the NIH (AI 21389). W.P.T. was supported in part by funding from the Georgia State University Research Program Enhancement program.

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